The Dictyostelium discoideum Rap1 signalling cascade and its functions during growth and development

Plak, Katarzyna

CHAPTER V
Dictyostelium RA domain containing proteins

Katarzyna Plak[1], Verena Kolsch[2], Peter JM van Haastert[1], Richard Firtel[2] and Arjan Kortholt[1]

[1]Department of Cell Biochemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands.

[2]Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0380, USA.

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Introduction

A large number of eukaryotic cellular signalling pathways depend on Ras family proteins. Due to regulatory GEF and GAP proteins, the Ras family members can rapidly shuttle between an active (GTP-bound) and inactive (GDP-bound) state. This property makes them key regulators in many signal transduction pathways (Wennerberg *et al.,* 2005).

Dictyostelium Ras family consists of 18 proteins and they have been studied for their multiple roles in vegetative growth, starvation induced chemotaxis, and multicellular development of *Dictyostelium* (Fey *et al.,* 2013; Wilkins and Insall, 2001). Rap1 protein is one of the members of this family and it had been shown to be important in processes as different as cell shape determination, attachment to the substratum, cell motility, production of phospholipids, and multicellular development (Rebstein *et al.,* 1993; Jeon *et al.,* 2007; Jeon *et al.,* 2009; Kortholt *et al.,* 2010; Parkinson *et al.,* 2009). Despite all these important roles, the downstream pathways of Rap1 are still not completely understood.

Only in the GTP-bound state Ras protein can interact with its downstream targets and thereby allows for a start of signalling cascades. Most Ras effectors contain either a Ras Binding Domain (RBD) or Ras Association (RA) domain that recognize GTP-bound Ras. Despite low amino acid sequence conservation, both type domains share similar, ubiquitin-like fold. The exposed, positively charged residues in the β1, β2 and α1 secondary structure elements of RA/RBD domain and negatively charged residues in the effector region of Ras are responsible for establishing the interaction (Kalhammer *et al.,* 1997; Wohlgemuth *et al.,* 2005). Based on sequence and fold similarity a large number of RBD/RA domain containing proteins can be identified in databases as SMART or PFAM (Letunic *et al.,* 2012; Punta *et al.,* 2012). It is however not possible to determine in silico if and to which Ras subfamily member the RA/RBD domain is binding (Kalhammer *et al.,* 1997; Wohlgemuth *et al.,* 2005). To understand the function of these RA/RBD domain proteins detailed biochemical characterization is necessary.

We recently performed a large proteomic screen to identify further interaction partners of *Dictyostelium* Rap1. Surprisingly, we did not find any RBD domain containing proteins in our proteomic screen and TalinB was the only RA domain containing protein found among the potential interactors. TalinB functions as an important regulator of

cellular adhesion and multicellular development and we have confirmed the direct interaction of Rap1 with the RA domain of TalinB (chapter 4). In addition we have showed the direct interaction and described the molecular function of another interactor; GxcC a RacGEF family member protein binds activated Rap1 via its ARM (Armadillo like) repeats containing domain (Plak *et al.,* 2013; see also chapter 3 of this thesis),

Until now more effort was concentrated on characterizing RBD proteins of *Dictyostelium* among which are phosphatidylinositide 3-kinases (Hoeller *et al.,* 2013), RIP3 (for studies on RIP3 binding specificity see thesis A. Khanna) and NdrC (Muller-Taubenberger *et al.,* 2014). Less is however known about the RA domain containing proteins in *Dictyostelium*. Only Phg2 has been characterized up to date and shown to be a downstream target of Rap1 (Kortholt *et al.,* 2006).

In this report we characterized *Dictyostelium* RA domain containing proteins for their Rap1 binding ability. Our results show that consistent with the data from our proteomic approach, none of the RA domains bind to active Rap1. Instead we show that RA domain containing protein DydA is a RasG effector which is important for *Dictyostelium* chemotaxis.

Results and Discussion

Dictyostelium RA domain containing proteins

Search for RA domain containing proteins in protein databases as SMART or PFAM reveals 3 *Dictyostelium* proteins with RA-domains (DDB_G0287875, DDB_G0284079 and DDB_G0280181) (Letunic *et al.,* 2012; Punta *et al.,* 2012). We used the sequences of these RA domains, the sequence of Phg2 RA domain and the sequence of RalGDS RA domain to search for other potential RA domain containing proteins in *Dictyostelium* proteome. We found 4 additional proteins with RA-like domains (Table 1).

Gene name	Protein name	Protein size (AA)	Potential Ra domain position (AA)
DDB_G0283699	Phg2	1387	(583–680)
DDB_G0287875	DydA	1211	(5-90), (1112-1196)
DDB_G0284079	-	303	(196-292)
DDB_G0280181	-	333	(225-310)
DDB_G0287505	TalB	2614	(1-77)
DDB_G0349530	-	1025	(4-97)
DDB_G0280081	Exoc2/sec5	1095	(406-508)
DDB_G0269362	FrmB	478	(46-126)

Table 1. *Dictyostelium* RA-like domain containing proteins.

It was shown previously that a conserved ubiquitin like fold, rather than amino acid sequence conservation is important for Ras-RA interaction (Kiel *et al.,* 2005; Wohlgemuth *et al.,* 2005). Consistently, the *Dictyostelium* RA domains and RalGDS-RA only show low sequence conservation, but all have the typical RA domain like fold (Fig 1). Positively charged amino acids located in the first two antiparallel β sheets and subsequent α helix secondary structures of the RA-fold are important for interaction with small G proteins (Wohlgemuth *et al.,* 2005). Six of the *Dictyostelium* RA domains contain these positive charged residues and are predicted to have this essential ββα structure fold (Fig. 1B) (Simossis *et al.,* 2005; Cole *et al.,* 2008). Since, the N-terminus of the Ra domain of DDB_G0280081 is predicted to form a β structure followed by a long α helix rather the conserved ββα structure fold, interaction with Ras is highly unlikely (Fig. 1B).

The RA proteins contain a wide variety of additional domains, including localization domains and domains that mediate protein – protein interactions (Fig. 2). The CH (Calponin Homology) domain was previously shown to mediate filamentous actin binding. FERM (4.1 protein/Ezrin/Redoxin/Moesin) domains have been implicated in localizing proteins to the plasma membrane, PH (Pleckstrin Homology) domains are well known for their binding to phosphoinositides, and ANK (Ankyrin repeats) are responsible for establishing protein- protein interactions (Letunic *et al.,* 2012; Punta *et al.,* 2012).

Figure 1

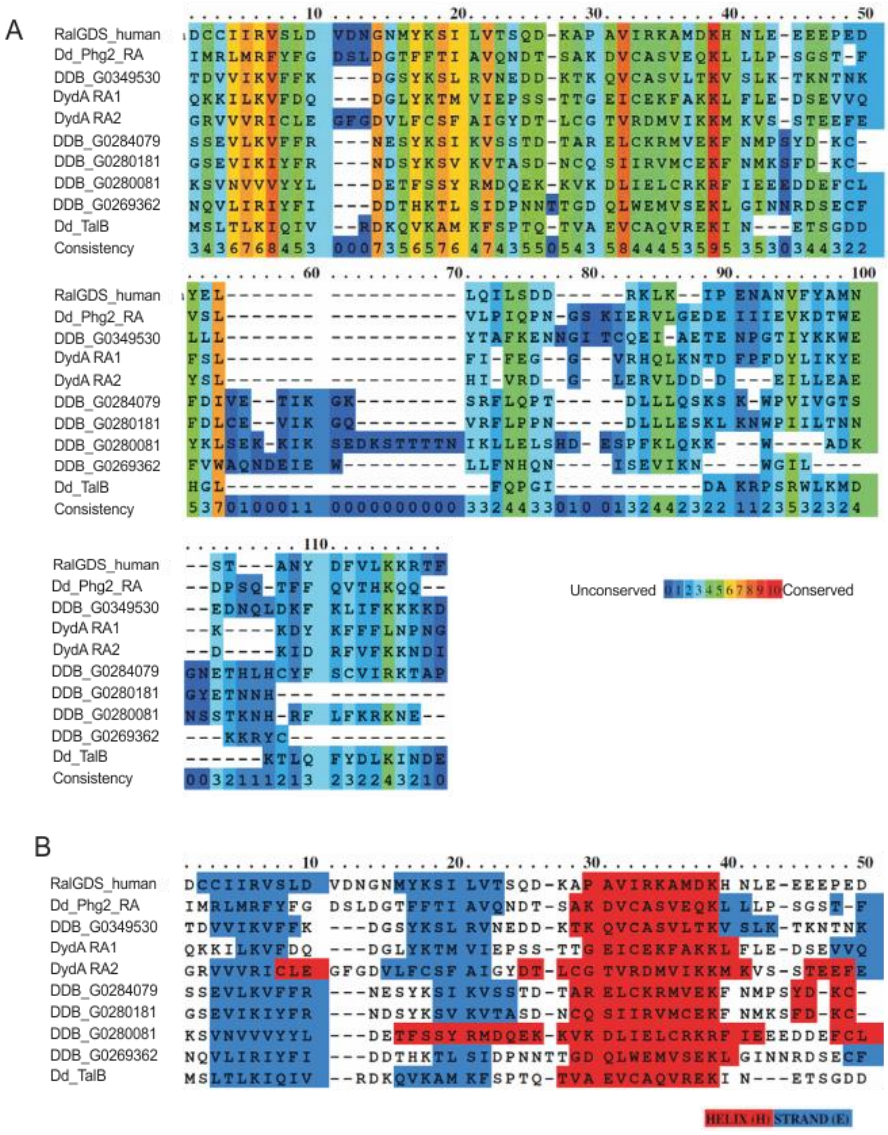

Figure 1. RA domains of Dictyostelium proteins show low sequence conservation but a conserved secondary structure fold.

(A) Potential RA domains of *Dictyostelium* have low aminoacid sequence conservation. Alignment of the RA domain sequences was created using PRALINE server. Colour scale indicates conservation scale of the given residue. **(B) Common RA domain secondary structure characterizes almost all potential RA domains of *Dictyostelium.*** Alignment of the RA domain sequences using PRALINE server. Colour scale indicates likelihood of forming α helix or β strand secondary structures for given sequences.

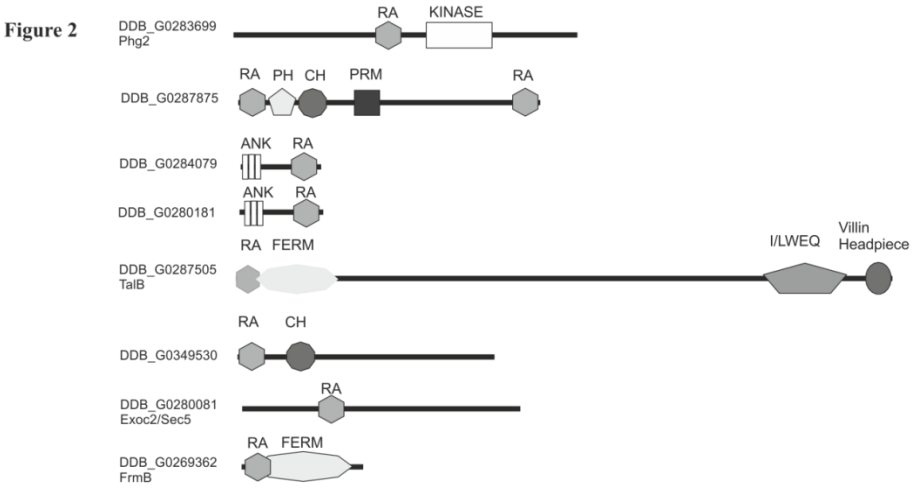

Figure 2. ***Domain composition of Dictyostelium RA proteins.***

RA (Ras-Association) domain, FERM (4.1 protein/Ezrin/Redoxin/Moesin) domain, I/LWEQ domain, PH (Pleckstrin Homology) domain, CH (Calponinn Homology) domain, PRM (Proline Rich Motif), ANK (Ankyrin Repeats) region

Only Phg2 and TalinB are true Rap1 effectors

Two RA proteins, namely Phg2 and TalinB were described before to bind specifically to active form of Rap1 (Kortholt *et al.,* 2006; chapter 4 of this thesis). Phg2 was described for its roles in regulating phagocytosis and cellular adhesion, processes also regulated by Rap1. TalinB was studied before for its important functions in regulating adhesion and multicellular development (Tsujioka *et al.,* 1999; Tsujioka *et al.,* 2008), and we were able to show that at least part of its functions depend on Rap1 binding.

The remaining RA domain containing proteins were not characterized up to date. To assess if these proteins also are able to bind to Rap1 we expressed the RA domains and full length proteins as GFP fusions in *Dictyostelium* and subsequently used these strains in a pull-down assay with Rap1-GppNHp protein as bait. We were unable to detect any binding between the RA proteins and active Rap1 using either full length or RA domain constructs in repetitive experiments (a representative set of pull-down experiments results is shown in Fig. 3). This result suggests that these proteins may be

involved in signal transduction downstream of other Ras family proteins of *Dictyostelium* or that the potential RA domains do not function as Ras Association domains at all. Below we characterize the binding specificity and function of DDB_G0287875 in more detail.

Figure 3

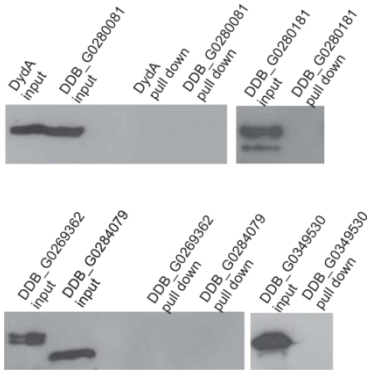

Figure 3. Pull- down experiment with GppNHp loaded GST-Rap1 and different RA domain containing proteins.

The RA domains or RA proteins were expressed as GFP fusion proteins and used as bait in a GST-Rap1 pull down. Consistently with the fact that none of this proteins was detected in our large scale proteomic screen, we could not detect any binding between those proteins and activated Rap1 in this directed approach.

DydA is a regulator of Dictyostelium chemotaxis.

DDB_G0287875 appears to be a member of the MRL family of adaptor proteins that act downstream of Ras-like GTPases and translate extracellular signals into changes of the actin cytoskeleton affecting cell motility and adhesion (Krause *et al.,* 2004; Lafuente *et al.,* 2004; Chang *et al.,* 2006; Lyulcheva *et al.,* 2008). Like other MRL proteins, DDB_G0287875 has an N-terminal RA domain, followed by a pleckstrin homology (PH) domain and a proline-rich motif (PRM). In addition, DDB_G0287875 has two calponin homology (CH) domains (Friedberg and Rivero, 2009) and a second RA domain at the C-terminus (Fig. 2). On the basis of the chemotactic defects of the DDB_G0287875 null strain described below, the protein was named Daydreamer, or DydA (Kolsch *et al.,* 2013).

To determine whether DydA plays a role in chemotaxis, we generated *dydA*−null cells by homologous recombination and analyzed the chemotactic properties of these cells. *dydA*−null cells exhibit strong chemotactic defects: when placed in a strong chemoattractant gradient emitted by a micropipette, *dydA*−null cells polarize weakly, move with reduced speed and deeply reduced directionality, and have more lateral filopodia (Fig. 4A). In shallow, linear gradients produced in a Dunn chamber, the cells do not move. The low, measured speed results from jiggling of the cells, which results in a change in the location of the cell's centroid (Table 2).

Role of the RA domains in controlling DydA function

To determine whether the DydA RA domains bind Ras and/or Rap1, similar to RA domains of other MRL members (Lafuente *et al.,* 2004), we expressed RA1 and RA2 as GFP fusions in wild-type cells and used the glutathione S-transferase (GST) fusions of the GDP and GTP forms of RasG, RasC, and Rap1, and found that GFP-RA1 was only pulled down by RasG-GTPγS from *Dictyostelium* cell lysates (Fig. 4B). We also did not observe binding of the activated form of Rap1 with a DydA fragment containing RA1 and the adjacent PH domain (unpublished data). None of the Ras proteins pulled down GFP-RA2. We also examined the ability of the activated (GppNHp-bound) form of recombinant GST-RasC, -RasG, -RasD, which is closely related to RasG, and -Rap1 to bind recombinant RA1 and RA2 and found that only RasG-GppNHp and RasD-GppNHp showed any detectable binding (Fig. 4B). Finally, we examined the ability of recombinant RA1 to stimulate the dissociation of mGppNHp from RasG, an assay of RA1 binding to RasG-GTP. In agreement with our other data indicating RasG-GTP binds RA1, we show that RA1 has GDI activity by demonstrating that addition of RA1 inhibits the dissociation of 2′/3′-O-(N-Methylanthraniloyl)-guanosine-5′-[(β,γ)-imido]triphos¬phate (mGppNHP) from RasG and that the inhibition depends upon the concentration of RA1 (Fig. 4C). As RasG is expressed throughout growth and aggregation, and RasD is preferentially expressed during multicellular development (Reymond *et al.,* 1984; Robbins *et al.,* 1989), we suggest that RasG is the major Ras protein that binds to DydA through RA1. We cannot exclude that another Ras protein we have not tested may interact with either RA domain.

Figure 4

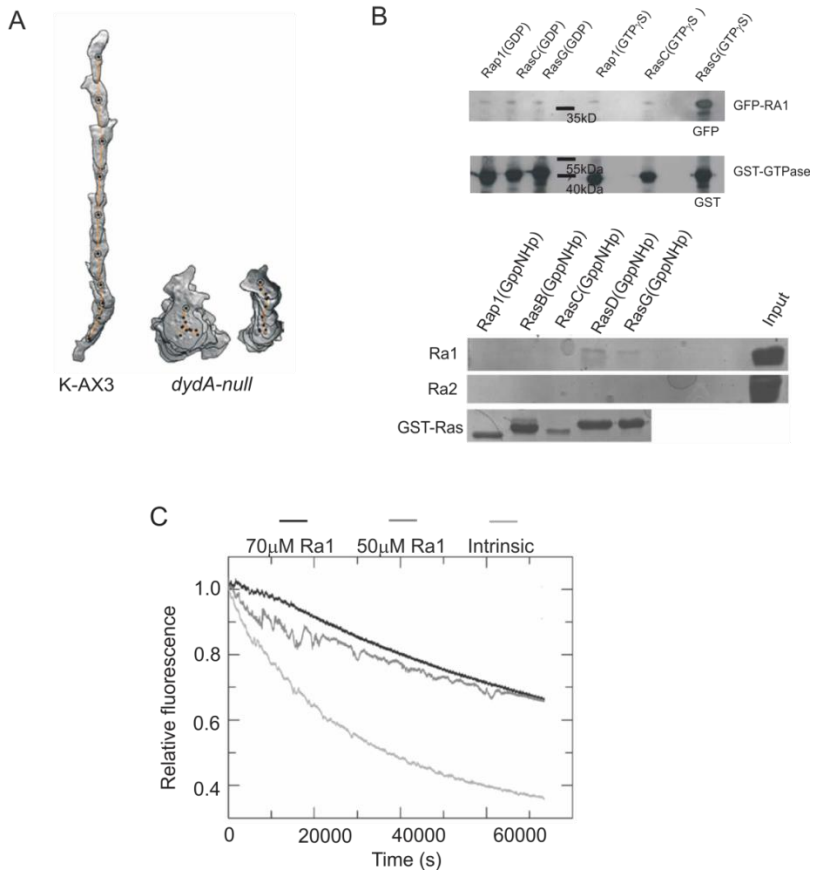

Figure 4. DydA is a RasG effector necessary for *Dictyostelium* chemotaxis.

(A). *dydA*-null cells are defective in cAMP chemotaxis. When dydA cells are exposed to cAMP gradient released from micropipette they fail to establish proper polarity and migrate towards the cAMP source as it is seen for the wild type control cells. **(B).** DydA binds to activated RasG and RasD, but not to Rap1, RasC, or RasB. To determine the specificity of DydA pull-down experiment was performed. Indicated GST-Ras proteins were prebound to GSH beads and circulated with GFP-RA protein containing cell lysates. Bound RA domains were detected by western blot against GFP tag. **(C).** RA domain of DydA specifically interacts with active RasG in the region of nucleotide binding pocket. Dissociation rate of mGppNHp from RasG was measured in the absence and presence of purified RA1 domain of DDB_G028787.

To further understand the role of the two RA domains, we examined the phenotypes of *dydA*−null cells expressing His-hemagglutinin-FLAG (HHF)-tagged DydA lacking both RA domains (DydAΔRA) and found that the cells exhibit strong chemotactic

phenotypes, indicating that the RA domains are required for DydA function. In a steep gradient emitted by a micropipette, the cells are less polarized and move slightly more slowly than wild-type cells. In a shallow, linear gradient produced in a Dunn chamber (Zicha *et al,* 1991), the cells are unable to migrate and the phenotype is as severe as that observed for *dydA*−null cells (Table 2). Both Dyd-GFP and DydAΔRA-GFP localizes to the leading edge of chemotaxing cells, suggesting that the RA domains are required for modulating the regulation of DydA function but not its localization per se.

Strain	Cells	Speed	Directionality
Wild type	16	7.85±0.96	0.81±0.052
dydA-null	17	2.30±0.33	0.072±0.098
dydA-null DydA OE	10	5.88±1.89	0.70±0.20
dydA-null DydA ΔRA	15	1.91±0.37	0.11±0.12

Table 2. *dydA* cells are defective in chemotaxis.

Wild type and mutant cells were starved and then exposed to gradients of cAMP. In shallow chemoatractant gradients wild type cells move efficiently towards the source of cAMP, *dydA*-null cells at the other hand showed severe defects in both their speed and directionality. This phenotype can be rescued by expression of full length DydA protein, but not the construct missing the RA domains.

Conclusions

In search for new Rap1 interactor partners we tested *Dictyostelium* RA domain containing proteins for their Rap1 binding abilities. DydA appeared a good candidate for a potential Rap1 effector as its domain architecture was similar to that of the MRL proteins, known regulators of cellular adhesion and motility (Colo *et al.,* 2012). However detailed biochemical studies showed that it is not binding to Rap1, but is an effector of RasG. The RasG/DydA interaction is crucial for proper chemotaxis of starved *Dictyostelium* cells, a function that depends on the presence of the RA domains. We showed that of all identified RA proteins, only Phg2 and TalinB are able to bind to activated Rap1 (see Chapter 4). Further research is necessary to determine if the other RA proteins are targets of other *Dictyostelium* Ras family proteins or, as was previously shown for several mammalian RA domains, are not able to bind to G-proteins at all (Kiel *et al.,* 2005). Importantly, our data

also shows that a non-biased approach is necessary to identify further components of the Rap pathway, as proteins with domains other than RA or RBD may interact specifically with Rap1 as is the case for RacGEF1 and GxcC (see Mun *et al.,* 2012 and Chapter 3 of this thesis). Our pull-down coupled to mass spectrometry approach will be important in this enterprise.

Materials and Methods

Strains and constructs.

Expression plasmids of the *Dictyostelium* RA domains and RA-domain containing proteins were created by means of PCR using the *Dictyostelium* cDNA library as a template (see table 3 and 4). PCR products were digested with the indicated restriction enzymes and ligated to *Dictyostelium* expression vectors pDM317 (N term GFP) and pDM323 (C term GFP) cut with BglII and SpeI. The final plasmids were electroporated to wild type *Dictyostelium* cell line. *Dictyostelium* cultures were grown on coated nunclon petri dishes (Nunc) or conical flasks on HL5-C medium (Formedium) in the presence of the appropriate antibiotics, 10 µg/ml Blasticidin S, 10 µg/ml Geneticin or 50 µg/ml Hygromycine B.

dydA knock out cell line was created by inserting bsr resistance cassette after the base pair position 561 of the dydA gene. The DydA overexpressor plasmid was created by cloning of *dydA* from the cDNA sequence and the *dydA*ΔRA plasmid was created by quick change mutagenesis (deletion of the base pairs: 13–270 and 3334–3558)

Primer	Sequence	RE
F DydA	GGATCCATGAAAAAGCGTGATAAATTAATTG	BamHI
R DydA	ACTAGTTTTTGATGACACAGGTCTATGATTTG	SpeI
F DDB_G0349530	GGATCCATGGGATCAACAGATGTTGTG	BamHI
R DDB_G0349530	GCTAGCTTGCTCCATCCATTTTACTTG	NheI
F DDB_G0284079	GGATCCATGTTAGAAAAATTTGATTATG	BamHI
R DDB_G0284079	ACTAGTTTGGAATTGTTCAACACATTCTG	SpeI
F DDB_G0280181	GGATCCATGATTAGTAATTCAAGTTATAGTC	BamHI
R DDB_G0280181	ACTAGTTAATTTAATTGCTTCTTTTGGAGCTG	SpeI
F DDB_G0280081	GGATCCATGTCTTCTTCAGATGATAC	BamHI
R DDB_G0280081	ACTAGTTAAAATTGGATCTAAATTAGCAG	SpeI
F DDB_G0269362	AGATCTATGGAATCATCATTTGAAGCATC	BglII
R DDB_G0269362	GCTAGCTTCTTTTGATTCCCATTCTTCAC	NheI

Table 3. Primers for *Dictyostelium* RA domain containing proteins.

Primer	Sequence	RE
F DydA RA1	GAGGATCCATGGAACCACCACAAAAAAAG	BamHI
R DydA RA1	GAGACTAGTCTTA ATCAAAACTAATGAATTCAC	SpeI
F DydA RA2	GAGGATCCATG ACACAAGGTAGAGTTGTG	BamHI
R DydA RA2	GAGCACTAGTCTTA TTTTGATGACACAGG	SpeI
F DDB_G0349530 RA	GGATCCATGGGATCAACAGATGTTGTG	BamHI
R DDB_G0349530 RA	ACTAGTAGTATTAGTTTGAGTTGATGCTG	SpeI
F DDB_G0284079 RA	AGATCTTCAGGTTATGATACAATTAGAG	BglII
R DDB_G0284079 RA	ACTAGTTTGGAATTGTTCAACACATTCTG	SpeI
F DDB_G0280181 RA	GGATCC ATATCACAACAACCAAATGCAAATTC	BamHI
R DDB_G0280181 RA	ACTAGTTAATTTAATTGCTTCTTTTGGAGCTG	SpeI
F DDB_G0280081 RA	GGATCCATGTCAAAATCTGTAAATGTTGTA	BamHI
R DDB_G0280081 RA	ACTAGTACCTGCTTTTGAATTGTTTACTGTG	SpeI
F DDB_G0269362 RA	AGATCTATGGAATCATCATTTGAAGCATC	BglII
R DDB_G0269362 RA	GCTAGCTTCCTCTGTATTACAATATC	NheI

Table 4. Primers for *Dictyostelium* RA domain constructs.

Pull-down experiments.

C-truncated GST Rap1 protein was purified as described previously (Kortholt *et al.,* 2006) and loaded with nonhydrolyzable GTP analogue (GppNHp) (Herrmann *et al.,* 1996). 50µg of the Rap1 protein was prebound to the GSH-agarose beads slurry and the beads were incubated for two hours with lysate of *Dictyostelium* cells expressing GFP protein of interest. Lysate was prepared by collecting 5x107 *Dictyostelium* cells and incubating them in Lysis Buffer (10 mM Na_2HPO_4 pH 7.2, 1% Triton X-100, 10% glycerol, 150mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF) with protease inhibitor mix (60µg/ml N-CBZPA, 100 µg/ml TAME, 80 µg/ml TPCK, 2 µg/m Pepstatin and 5 µg/ml Leupeptin) for 10 minutes at 4˚C and subsequent centrifugation at 16000g 10min 4˚C. After the beads slurry was incubated with *Dictyostelium* cell lysates the beads were washed 3 times and the bound proteins were eluted by boiling in 1xSDS buffer (0.5% β-mercapto-ethanol, 0.5% SDS, 50mM Tris-HCl pH 6.8, 12.5% glycerol and 0.04% bromphenol blue). The bound RA proteins were detected by Western blot against GFP tag.

Purification of GST-tagged recombinant RA domain of DydA

The RA domain sequence was amplified from *Dictyostelium* cDNA library by means of PCR, digested with BamHI and ligated to pGEX-4T vector for Bacterial expression. *E. coli* Rosetta cells were used as expression system. The cells were first grown at 37˚C to an OD600 of 0.6 and subsequently induced with 0,1mM IPTG (Isopropyl-1-thio-β-D-galactopyranoside) and further grown ON at 22˚C. Harvested cells were washed in 0,6% NaCl solution and lysed by sonication in lysis buffer (50mM Tris, 50mM NaCl, 5mM DTT, 5mM $MgCl_2$, pH 7,5) with Proteinase inhibitor mix (60µg/ml N-CBZPA, 100 µg/ml TAME, 80 µg/ml TPCK, 2 µg/m Pepstatin and 5 µg/ml Leupeptin). The sample was then precleared by centrifugation (45min, 4˚C, 75600g) and soluble fraction was loaded on lysis buffer equilibrated GSH affinity column (GE-Healthcare). Unbound proteins were removed by washing with High Salt Lysis buffer (50mM Tris, 500mM NaCl, 5mM DTT, 5mM $MgCl_2$, pH 7,5) and bound proteins eluted with Gluthathione buffer (50mM Tris, 50mM NaCl, 5mM DTT, 5mM $MgCl_2$ and 20mM Glutathione, pH7,5). The

obtained sample was concentrated on 10kDa cutoff centrifugation filters (Milipore) and purity of the protein was confirmed by SDS page.

GDI experiment

GDI assays were performed as described before (Herrmann *et al,* 1996). mGppNHp or mGDP preloaded Ras or Rap proteins were incubated at 25˚C in assay buffer (50 mM Tris-HCL, pH 7.5, 5mM $MgCl_2$, 50 mM NaCl and 5 mM DTE) in the presence of excess of unlabeled nucleotide in a Spex spectrofluorometer (Spex Industries). The decay in observed fluorescence was measured in the presence and absence of various concentration of purified RA domain of DydA and the results were plotted using GRAPHITE software.

Chemotaxis assays

Chemotaxis assays and analysis were performed as described before (Chung and Firtel 1999). Shortly, *Dictyostelium* cells were harvested and pulsed with 30 nM cAMP for 5 hours at room temperature. Subsequently cells were allowed to settle at the glas bottom dishes (MatTek) and a micropipette filled with 150µM cAMP was positioned in the field of cells. The chemotaxic behaviour was monitored by bright field microscopy and movies were analysed with DIAS software (Wessels and Soll, 1998).

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